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Structure-Activity Relationship Study of the Plant-Derived Decapeptide OSIP108 Inhibiting *Candida albicans* Biofilm Formation

Nicolas Delattin,[a] Katrijn De Brucker,[a] David J. Craik,[b] Olivier Cheneval,[b] Barbara De Coninck,[a] Bruno P. A. Cammue,[a,c] Karin Thevissen[a]

Centre of Microbial and Plant Genetics, KU Leuven, Leuven, Belgium[a]; Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia[b]; Department of Plant Systems Biology, Vlaams Instituut voor Biotechnologie, Ghent, Belgium[c]

We performed a structure-activity relationship study of the antibiofilm plant-derived decapeptide OSIP108. Introduction of positively charged amino acids R, H, and K resulted in an up-to-5-fold-increased antibiofilm activity against *Candida albicans* compared to native OSIP108, whereas replacement of R9 resulted in complete abolishment of its antibiofilm activity. By combining the most promising amino acid substitutions, we found that the double-substituted OSIP108 analogue Q6R/G7K had an 8-fold-increased antibiofilm activity.

Disseminated candidiasis is associated with high mortality rates, especially in patients immunocompromised due to HIV and in patients who have received immunosuppressive drugs for cancer therapy or organ transplantation (1). Moreover, in natural environments, *Candida* spp. are mainly found in biofilms. Biofilms are well-structured microbial populations that are attached to a biotic (e.g., the human body) or abiotic (e.g., medical device) surface and are surrounded by a self-produced extracellular matrix of polysaccharides. Such biofilms are characterized by an increased resistance toward the human immune system and the currently available antimycotics (2, 3). Hence, *C. albicans* biofilms are considered critical in the development of fungal infections and their clinical outcome (2, 4, 5). Moreover, biofilm formation is related to chronic infections with *Candida* spp. (6). From the currently available antimycotics, only lipid formulations of amphotericin B and the echinocandins, such as caspofungin, are active against fungal biofilms (7). However, resistance against these antifungal agents has been described (8–12), urging the identification of new antibiofilm agents.

We previously identified the *Arabidopsis thaliana*-derived decapeptide OSIP108 (13), which specifically interferes with the biofilm formation process of *C. albicans* without affecting cell viability (14). The latter is an important characteristic to potentially limit the incidence of resistance. Furthermore, OSIP108 synergistically interacts with amphotericin B and caspofungin against mature *C. albicans* biofilms (14). A preliminary structure-activity relationship study of OSIP108 showed that (i) the order of amino acid residues is important for antibiofilm activity, as a scrambled version (S-OSIP108) containing all amino acids of OSIP108 but in a randomized order showed no antibiofilm activity, (ii) OSIP108 containing all amino acids in the D-configuration (D-OSIP108) still exhibits antibiofilm activity, and (iii) cyclization of OSIP108 is not favorable for its antibiofilm activity (14). In this follow-up study, we performed a whole amino acid scan of OSIP108, in which every amino acid of OSIP108 was individually replaced by all 19 other common amino acids (190 OSIP108 analogues). The aim of this study was to identify important structural determinants for OSIP108 antibiofilm activity as a basis to develop OSIP108 analogues with improved antibiofilm activity compared to native OSIP108.

The 190 peptide analogues of OSIP108 (MLCVLQGLRE) were ordered from Pepscan (Lelystad, The Netherlands) and were of crude purity, and the abilities to inhibit biofilm formation of *C. albicans* SC5314 (at 0.39 to 50 μM) were assessed as described previously (14). BIC-2 values, i.e., the minimal peptide concentrations that reduced the metabolic activity of the biofilms by 50% (14), were determined relative to the growth control (0.5% dimethyl sulfoxide), and the fold change in the BIC-2, relative to the native OSIP108 peptide, was calculated. The constructed heat map (Fig. 1) contains the average fold change in BIC-2s (increased or decreased activity compared to native OSIP108) of at least two independent biological experiments consisting of at least duplicate measurements. For all the individual amino acids from the native OSIP108 sequence, the peptide analogues were ranked from lowest to highest antibiofilm activity (Fig. 1).

Statistical analysis (Table 1) was performed using GraphPad Prism 6 software (San Diego, CA) via a one-way analysis of variance using Bonferroni's multiple comparison test, with the average BIC-2s of the OSIP108 analogues compared with the average BIC-2 of native OSIP108.

From this heat map, it is clear that replacement of the glycine at position 7 (G7) with 13 out of the 19 amino acids, irrespective of the functional nature of the amino acid, resulted in at least 1.5-fold-increased antibiofilm activity compared to native OSIP108. Being the only amino acid without a side chain, G allows flexibility of the peptide conformation. So, it seems that peptides that are more conformationally restrained exert a better antibiofilm activity. To investigate this hypothesis further, we tested two OSIP108 analogues in which the G7 was replaced by a D-amino acid, namely, G7-D-histidine (G7-DH) and G7-D-lysine (G7-DK), as these D-amino acids potentially occupy a different conformational space than do the L-amino acids (Table 1). Both would result in a similar loss of flexibility to their L-counterparts, but they would

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Address correspondence to Bruno P. A. Cammue, bruno.cammue@biw.kuleuven.be.

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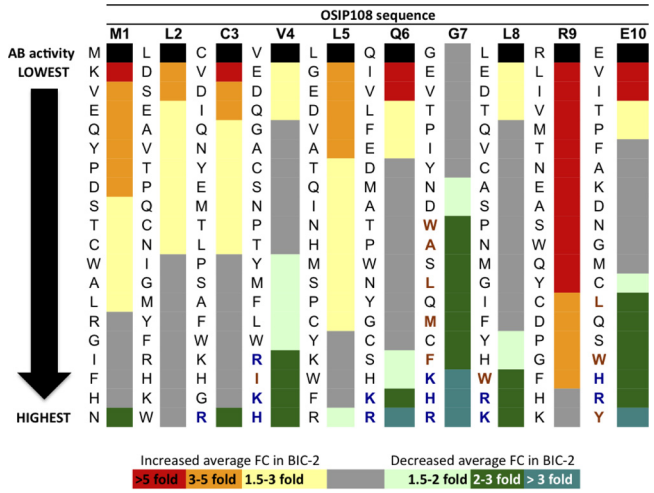

FIG 1 Results of the structure-activity relationship study of OSIP108. *C. albicans* biofilms were grown in the presence of OSIP108 analogues in which every amino acid of the OSIP108 sequence was individually replaced with the indicated amino acid, and their antibiofilm (AB) activities were determined. Colors indicate average fold changes (FC) in BIC-2s (increased or decreased) relative to the native OSIP108 in at least two biologically independent experiments consisting of at least duplicate measurements. Black, native sequence. For every amino acid of OSIP108, analogues are ranked from lowest (top) to highest (bottom) antibiofilm activity. Amino acids marked in blue are positively charged amino acids; amino acids in brown are amino acids with a hydrophobic side chain.

place the side chains in different locations. Since the antibiofilm activities of these peptide analogues were not statistically different from that of the native OSIP108 ($P > 0.05$) (Table 1), it appears that neither the nature nor the location of the side chain is important at position 7. Furthermore, replacement of valine 4 (V4) and glutamic acid 10 (E10) with at least 8 other amino acids resulted in increased antibiofilm activity of OSIP108 compared to native OSIP108 (Fig. 1). All these data indicate that most OSIP108 analogues with improved antibiofilm activity can be obtained by replacing G7, V4, or E10. In contrast, replacement of the arginine 9 (R9) with 17 out of the 19 amino acids led to at least a 3-fold reduction of the antibiofilm activity compared to native OSIP108, showing the absolute importance of R9 (Fig. 1). Interestingly, the only two OSIP108 analogues in which an R9 substitution resulted in activity comparable to the native OSIP108 were the analogues where the positively charged R was replaced by one of the other two positively charged amino acids, histidine (H) and lysine (K) (Fig. 1). These data indicate that the presence of a positively charged amino acid at the ninth position of the OSIP108 sequence is essential for its antibiofilm activity. Finally, as can be seen from Fig. 1, methionine 1 (M1), leucine 2 (L2), cysteine 3 (C3), and L5 are also important for antibiofilm activity, although to a lesser extent than R9. In agreement with this finding, we found that an OSIP108 dimer that was formed via disulfide bonds of the C3 side chains showed no antibiofilm activity (BIC-2, >100 μM) (data not shown).

In general, it is clear that the antibiofilm activity of OSIP108 can be increased at least 2-fold by (i) the introduction of positively charged amino acids, such as H and/or K and/or R at C3, V4, glutamine 6 (Q6), G7, L8, and E10, and/or by (ii) the introduction of amino acids with a hydrophobic side chain at V4 (isoleucine [I]), G7 (tryptophan [W], alanine [A], L, M, or phenylalanine [F]), L8 (W), or E10 (L, W, or tyrosine [Y]) (Fig. 1). In line with these observations, introduction of negatively charged amino acids, such as aspartic acid (D) and/or E at M1, L2, C3, or L5, resulted in at least a 3-fold-reduced antibiofilm activity of OSIP108. We previously demonstrated that OSIP108 mainly localizes to the cell surface of *C. albicans* yeast and hyphal cells (14). The *C. albicans* cell surface has an overall negative charge due to the presence of phosphodiester bridges in the carbohydrate side chains and the carboxyl groups of the cell wall proteins (15, 16). Therefore, the introduction of positively charged amino acids at various places in the OSIP108 sequence and removal of the negatively charged E10 may enhance the interaction of OSIP108 with its yet-unidentified cell wall target(s).

Next, we selected the 5 most promising peptide analogues, i.e., those with a BIC-2 at least 3-fold lower than the native OSIP108, from the screening, namely, Q6R (Q6 replaced by R), G7H, G7K, G7R, and E10Y (Fig. 1; Table 1). To assess whether we could further increase the antibiofilm activities of these OSIP108 derivatives, we combined these substitutions in double- and triple-substituted analogues and determined the BIC-2s of these OSIP108 analogues against *C. albicans* biofilms (Table 1). We found that the antibiofilm activities of various double OSIP108 analogues, namely, Q6R/G7K, Q6R/G7R, and G7R/E10Y, could be additionally improved compared to the selected single-substituted OSIP108 analogues. For example, the antibiofilm activity of Q6R/G7K was increased 8.1-fold above that of native OSIP108, whereas the Q6R and G7K single-substituted analogues were characterized by 4.8- and 3.7-fold-increased antibiofilm activities, respectively, compared to native OSIP108 (Table 1). Surprisingly, combination of the improved analogue E10Y with either Q6R or G7K (leading to Q6R/E10Y and G7K/E10Y, respectively) resulted

TABLE 1 Antibiofilm activities of selected OSIP108 analogues against *C. albicans* biofilms[a]

OSIP108 analogue	Sequence	BIC-2 (mean ± SEM)	FC
OSIP108	MLCVLQGLRE	8.1 ± 1.1	NA
Q6R	MLCVL**R**GLRE	1.7 ± 0.3	4.8
G7H	MLCVLQ**H**LRE	2.5 ± 0.4	3.2
G7K	MLCVLQ**K**LRE	2.2 ± 0.4	3.7
G7R	MLCVLQ**R**LRE	2.1 ± 0.3	3.9
E10Y	MLCVLQGLR**Y**	2.3 ± 0.2	3.5
G7-D-H[#]	MLCVLQ(**D-H**)LRE	2.9 ± 0.0	2.8
G7-D-K[#]	MLCVLQ(**D-K**)LRE	2.9 ± 0.0	2.8
Q6R/G7H	MLCVL**RH**LRE	1.9 ± 0.2	4.3
Q6R/G7K	MLCVL**RK**LRE	1.0 ± 0.0	8.1
Q6R/G7R	MLCVL**RR**LRE	1.3 ± 0.1	6.2
Q6R/E10Y	MLCVL**R**FLR**Y**	> 25	NA
G7H/E10Y[#]	MLCVLQ**H**LR**Y**	5.1 ± 0.6	1.6
G7K/E10Y	MLCVLQ**K**LR**Y**	>25	NA
G7R/E10Y*	MLCVLQ**R**LR**Y**	1.5 ± 0.2	5.4
Q6R/G7H/E10Y*	MLCVL**RH**LR**Y**	1.4 ± 0.3	5.8
Q6R/G7K/E10Y	MLCVL**RK**LR**Y**	>25	NA
Q6R/G7R/E10Y	MLCVL**RR**LR**Y**	>25	NA

[a] Amino acid replacements are indicated in boldface. Analogues with paradoxical biofilm effects are indicated with an asterisk. Analogues with increased activity were further characterized by the fold change (FC) relative to OSIP108 (FC = BIC-2 for OSIP108/BIC-2 OSIP108 for analogue). NA, not applicable. All peptide analogues were statistically different ($P < 0.05$) from the native OSIP108 except for the analogues indicated with a pound sign.

in a total abolishment of the antibiofilm activity (Table 1; BIC-2, >25 μM). Next, we assessed the antibiofilm activities of the triple-substituted analogues Q6R/G7H/E10Y, Q6R/G7K/E10Y, and Q6R/G7R/E10Y. The antibiofilm activity of Q6R/G7H/E10Y was increased 5.8-fold above that of native OSIP108 and thus slightly better than the corresponding single-substituted OSIP108 analogues (resulting in up to a 4.8-fold-increased antibiofilm activity compared to OSIP108). Two of them, namely, Q6R/G7K/E10Y and Q6R/G7R/E10Y, showed no antibiofilm activity (Table 1; BIC-2, >25 μM). As already observed for the double analogues, combining single-amino acid substitutes with improved antibiofilm activities does not necessarily result in further-increased antibiofilm activity and, on the contrary, could completely abolish it.

As the highest antibiofilm activity was observed for the Q6R/G7K analogue, we synthesized this OSIP108 analogue, as well as a cyclic derivative of this double-substituted analogue (which in view of potential applications is expected to be more stable than the corresponding linear peptide) with 99% purity, as previously described (14). The antibiofilm activity of the 99% pure double-substituted Q6R/G7K analogue was not different from that of the crude version (BIC-2, 1.5 ± 0.2 μM versus 1.0 ± 0.0 μM, respectively [means ± standard errors of the means]). Furthermore, in line with our previous findings that cyclization is not favorable for OSIP108 antibiofilm activity (14), the cyclic Q6R/G7K analogue showed no antibiofilm activity (BIC-2, >25 μM).

Strikingly, the single-substituted OSIP108 analogues E10F, E10M, E10K, E10H, and E10R and the double-substituted G7R/E10Y and triple-substituted Q6R/G7H/E10Y OSIP108 analogues allowed paradoxical biofilm formation at higher concentrations (≥6.25 or 12.5 μM) (data not shown). Paradoxical biofilm formation is defined as a resurgence of biofilm formation (≥50% relative to control treatment) at peptide concentrations above the BIC-2 (17). A similar paradoxical effect of caspofungin has been observed on *C. albicans* biofilms and planktonic cells (17, 18); this effect was found to be associated with multiple cell wall rearrangements and calcineurin-mediated signaling pathways in planktonic *C. albicans* cells (18, 19). Wiederhold and colleagues showed higher *MKC1* expression in *C. albicans* planktonic cells treated with caspofungin concentrations leading to this paradoxical growth phenomenon compared to fungicidal caspofungin concentrations (19). *MKC1* encodes the central kinase of the *C. albicans* cell wall integrity (CWI) pathway (20). We previously showed that OSIP108 activates the *C. albicans* cell wall integrity pathway (14). However, such a paradoxical biofilm effect was not observed for the native OSIP108. It remains to be elucidated whether the OSIP108 analogues that induce this paradoxical growth phenomenon in *C. albicans* biofilm cells induce the CWI pathway to a greater extent than native OSIP108 and whether this induction of the CWI pathway is responsible for the observed paradoxical biofilm effect.

In conclusion, this study shows that site-specific amino acid substitutions can significantly alter the antibiofilm activity of OSIP108. Subsequent double and triple combinations of analogues with improved antibiofilm activities allowed us to select OSIP108 with Q6R/G7K as the tested analogue with highest antibiofilm potential, with an 8.1-fold-higher activity against *C. albicans* biofilms. In view of the urgent clinical need for novel and more valuable antibiofilm treatments, the OSIP108 variants with improved antibiofilm activities are valuable antibiofilm lead molecules.

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